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RNA-Mediated Silencing in Algae: Biological Roles and Tools for Analysis of Gene Function▽

Heriberto Cerutti,[1]* Xinrong Ma,[1] Joseph Msanne,[2] and Timothy Repas[1]

School of Biological Sciences and Center for Plant Science Innovation[1] and School of Natural Resources,[2] University of Nebraska, Lincoln, Nebraska

Algae are a large group of aquatic, typically photosynthetic, eukaryotes that include species from very diverse phylogenetic lineages, from those similar to land plants to those related to protist parasites. The recent sequencing of several algal genomes has provided insights into the great complexity of these organisms. Genomic information has also emphasized our lack of knowledge of the functions of many predicted genes, as well as the gene regulatory mechanisms in algae. Core components of the machinery for RNA-mediated silencing show widespread distribution among algal lineages, but they also seem to have been lost entirely from several species with relatively small nuclear genomes. Complex sets of endogenous small RNAs, including candidate microRNAs and small interfering RNAs, have now been identified by high-throughput sequencing in green, red, and brown algae. However, the natural roles of RNA-mediated silencing in algal biology remain poorly understood. Limited evidence suggests that small RNAs may function, in different algae, in defense mechanisms against transposon mobilization, in responses to nutrient deprivation and, possibly, in the regulation of recently evolved developmental processes. From a practical perspective, RNA interference (RNAi) is becoming a promising tool for assessing gene function by sequence-specific knockdown. Transient gene silencing, triggered with exogenously synthesized nucleic acids, and/or stable gene repression, involving genome-integrated transgenes, have been achieved in green algae, diatoms, yellow-green algae, and euglenoids. The development of RNAi technology in conjunction with system level "omics" approaches may provide the tools needed to advance our understanding of algal physiological and metabolic processes.

Algae are a large group of aquatic, characteristically photoautotrophic, eukaryotic organisms. However, despite some shared features, like an aquatic lifestyle, the capacity to carry out photosynthesis in membrane-bound chloroplasts, and the lack of specialized organs characteristic of land plants, algae are very diverse phylogenetically (4, 9, 69, 70, 96). They include organisms derived from a primary endosymbiotic event (a heterotrophic eukaryote engulfing a photosynthetic cyanobacterium), as well as those derived from subsequent secondary or even tertiary endosymbiotic events (nonphotosynthetic or photosynthetic eukaryotes engulfing photosynthetic eukaryotes) (70). As a consequence of this complex evolutionary history, algae are distributed within four of the six major eukaryotic supergroups, namely, the Archaeplastida, Chromalveolata, Rhizaria, and Excavata (Table 1) (4, 9, 69, 70, 96). Regardless of whether these supergroups are monophyletic (4, 9, 69), it is clear that some algae are more closely related to apicomplexan parasites (Chromalveolata), for instance, than to land plants (Archaeplastida) (70).

Algal species play important roles in marine, freshwater, and even terrestrial ecosystems. Notably, 30 to 50% of the planetary net photosynthetic productivity (the difference between autotrophic gross photosynthesis and respiration) is of marine origin and dependent on phytoplankton biomass (8, 26). The great potential of microalgae as feedstocks for renewable biofuel production is also gaining recognition (38, 75, 94). Yet, despite the recent sequencing of several algal genomes (3, 6, 7, 17, 21, 31, 57, 59, 68, 70, 74, 95, 96), we still know very little about the basic biology of most algae. In particular, gene regulatory mechanisms, presumably involving networks of transcription factors and noncoding RNAs, remain largely unexplored. Progress in this area will benefit greatly from the functional characterization of novel genes and from system level approaches aimed at understanding and modeling the biochemical and regulatory networks that control algal physiological and metabolic features. In this context, greater knowledge of RNA-mediated silencing pathways in algae may provide insights into both a mechanism(s) of gene regulation and a tool for the characterization of genes with unknown functions.

RNA-mediated silencing is an evolutionarily conserved process by which small RNAs (sRNAs; ~20 to 30 nucleotides in length) induce the inactivation of cognate sequences via a variety of mechanisms, including translation inhibition, RNA degradation, and/or transcriptional repression (10, 14, 25, 30, 45, 53, 92). Intriguingly, recent results indicate that sRNAs may also participate in the activation of gene expression (50, 84). At least two major classes of sRNAs have been identified in many eukaryotes: microRNAs (miRNAs) and small interfering RNAs (siRNAs) (5, 10, 30, 45, 53, 92). miRNAs generally originate from endogenous, single-stranded noncoding RNA transcripts or introns that fold into imperfect stem-loop structures. They often modulate the expression of genes with roles in development, physiological or metabolic processes, or stress responses (5, 10, 18, 25, 30, 45, 49, 92). siRNAs are produced from long, near-perfect-complementarity double-stranded RNAs (dsRNAs) of diverse origins, including the

* Corresponding author. Mailing address: School of Biological Sciences and Center for Plant Science Innovation, University of Nebraska—Lincoln, E211 Beadle Center, P.O. Box 880666, Lincoln, NE 68588-0666. Phone: (402) 472-0247. Fax: (402) 472-8722. E-mail: hcerutti1@unl.edu.

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TABLE 1. Distribution of core RNAi machinery components, small RNAs, and experimentally induced RNAi in algal species[g]

Species or strain	Genome size (Mb)	AGO-Piwi like	Dicer like	RDR like	miRNAs/siRNAs	RNAi	Reference(s)
Archaeplastida							
Green algae							
Ostreococcus tauri	12.5	−	−	−	U	U	21
Ostreococcus lucimarinus	13.2	−	−	−	U	U	68
Micromonas sp. strain RCC299	20.9	+ (1)	−	−	U	U	95
Micromonas sp. strain CCMP1545	21.9	−	−	−	U	U	95
Chlorella variabilis NC64A	46.0	+ (1)	+ (1)	−	U	U	6
Coccomyxa sp. strain C-169[a]	49.0	+ (2)	?	+ (1)	U	U	
Chlamydomonas reinhardtii	120.0	+ (3)	+ (3)	−	Y	Y	46, 59, 79
Volvox carteri[b]	138.0	+ (2)	+ (1)	−	U	Y	16, 24, 74
Dunaliella salina	300.0	U	U	U	U	Y	44, 85
Red algae							
Cyanidioschyzon merolae	16.5	−	−	−	U	U	57
Porphyra yezoensis[b]	260.0	U	U	U	Y	U	52, 56
Chromalveolata							
Stramenopiles							
Diatoms							
Phaeodactylum tricornutum	27.4	+ (1)	?	+ (1)	Y	Y	7, 22, 77
Thalassiosira pseudonana	32.4	+ (1)	?	+ (2)	U	U	3
Fragilariopsis cylindrus[c]	80.5	+ (2)	+ (2)	?	U	U	
Brown alga *Ectocarpus siliculosus*[b]	214.0	+ (1)	+ (1)	+ (2)	Y	U	17
Yellow-green alga *Vaucheria frigida*[d]	U	U	U	U	U	Y	87
Pelagophyte *Aureococcus anophagefferens*	56.0	−	−	−	U	U	31
Haptophyte *Emiliania huxleyi* CCMP1516[e]	168.0	+ (1)[f]	?	+ (3)[f]	U	U	
Rhizaria, chlorarachniophytes	U	U	U	U	U	U	
Excavata, euglenoid *Euglena gracilis*	U	U	U	U	U	Y	42, 43

[a] http://genome.jgi-psf.org/Coc_C169_1/Coc_C169_1.home.html.
[b] Multicellular algal species.
[c] http://genome.jgi-psf.org/Fracy1/Fracy1.home.html.
[d] Coenocytic filamentous alga.
[e] http://genome.jgi-psf.org/Emihu1/Emihu1.home.html.
[f] Possibly additional genes in the genome; evaluation limited by incomplete models in draft genome.
[g] Abbreviations: −, gene absent from the genome; +, gene present in the genome (number of encoding genes indicated in parentheses); ?, gene possibly present in the genome but fairly divergent from canonical models; U, unknown; Y, occurrence of miRNAs/siRNAs or of experimentally induced RNAi.

hairpin transcripts of long inverted repeats (IRs), the products of convergent transcription or RNA-dependent RNA polymerase (RDR) activity, viral and transposon RNAs, or dsRNAs experimentally introduced into cells (10, 14, 30, 45, 53, 92). These siRNAs play various roles in the posttranscriptional regulation of gene expression, suppression of viruses and transposable elements, DNA methylation, heterochromatin formation, and/or DNA elimination (10, 14, 30, 45, 54, 92). The function of long dsRNAs in triggering gene silencing was initially characterized in *Caenorhabditis elegans* and termed RNA interference (RNAi) (27). Yet, in slightly over a decade, RNAi has evolved from a fascinating biological phenomenon into a powerful experimental tool for reverse genetics studies and for inducing phenotypic changes for practical applications (33, 35, 79).

RNA-mediated silencing pathways have been studied in the unicellular green alga *Chlamydomonas reinhardtii* and used as a reverse genetics tool in a few algal species. However, RNAi mechanisms and their applications remain largely uncharacterized in most algae. This review will examine the existence of core components of the RNAi machinery in algae, as well as elaborate on the known or inferred biological role(s) of RNA-mediated silencing in these aquatic organisms. We will also discuss briefly the usefulness of RNAi as a tool to study gene function, an ever-increasing challenge as additional algal genomes become available and the number of genes with uncharacterized roles keeps growing.

RNAi MACHINERY IN ALGAE

Biochemical and genetic studies of multiple eukaryotes have led to the identification of three key components of the RNAi machinery, namely, Dicer, Argonaute-Piwi (AGO-Piwi), and RDR (10, 14, 25, 30, 45, 53, 92). Hairpin and long dsRNAs are

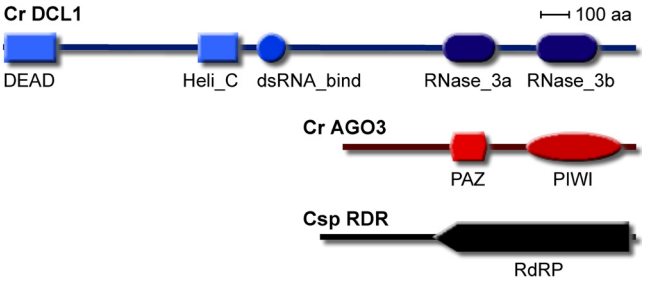

FIG. 1. Domain architecture of core components of the RNAi machinery in green algae. Protein sequences of *Chlamydomonas reinhardtii* Dicer-like 1 (Cr DCL1) and Argonaute 3 (Cr AGO3), as well as of *Coccomyxa* sp. strain C-169 RDR (Csp RDR), were examined for the presence of conserved domains by comparison with the SMART and PFAM databases. Polypeptide diagrams are shown to scale. DEAD, DEXD/H-like helicase domain; Heli_C, helicase C-terminal domain; dsRNA-bind, dsRNA binding fold (DUF283); RNase_3a/b, RNase III catalytic domains; PAZ, Piwi-Argonaute-Zwille domain; PIWI, a complex domain consisting of two structural subdomains termed MID and Piwi, the latter resembling an RNase H fold; RdRP, eukaryotic-type RDR domain; aa, amino acids.

processed into sRNAs (~20 to 30 nucleotides in length) by an RNase III-like endonuclease named Dicer (10, 14, 53, 92) (Fig. 1). These sRNAs are then incorporated into effector complexes, which include members of the AGO-Piwi family of proteins. This family consists of two main classes of closely related polypeptides, one named after *Arabidopsis thaliana* AGO1 and the other named after *Drosophila melanogaster* Piwi (14, 37). AGO-Piwi proteins contain a PAZ (Piwi-Argonaute-Zwille) domain, which binds to the 3′ ends of sRNAs, and a Piwi domain, which is structurally related to RNase H (10, 14, 37, 53) (Fig. 1). Some AGO-Piwi polypeptides function as sRNA-guided endonucleases ("slicers") that cleave complementary transcripts, whereas others appear to lack endonucleolytic activity and may be part of effector complexes involved in nondegradative RNAi processes such as translation repression (10, 14, 25, 30, 37, 39, 45, 53, 91, 92, 97). In certain eukaryotes, RDRs also play an important role in RNAi (14, 15, 51, 67) (Fig. 1). In these organisms, RDR activity may initiate RNAi by producing dsRNA from single-stranded transcripts or dramatically enhance the RNAi response by amplifying the precursors of sRNAs (14, 15, 51, 67).

To assess the existence of the RNAi machinery in algal species, we have identified polypeptides related to AGO-Piwi, Dicer, or RDR by BLAST or PSI-BLAST searches of protein and/or translated genomic DNA databases corresponding to several phylogenetically diverse algae (Table 1). Since some of the genomes examined are in the draft stage, an important caveat to our analyses is that some proteins may be missing from the databases whereas others may have errors in the predicted gene structure. However, we considered as potential homologs only polypeptides that exhibited enough sequence similarity to be aligned together (E value, based on Smith-Waterman alignments, <1E-5) and to be used for phylogenetic tree reconstruction. Based on these criteria, homologs of key RNAi machinery components and/or evidence of experimentally induced RNAi activity were observed in algal species belonging to three eukaryotic supergroups (Table 1). Yet, as previously reported for *Saccharomyces cerevisiae* and *Trypanosoma cruzi* (11, 14, 23), core RNAi machinery components seem to be entirely absent from several algae with small nuclear genomes such as the red alga *Cyanidioschyzon merolae* and the green algae *Ostreococcus lucimarinus*, *Ostreococcus tauri*, and *Micromonas* sp. strain CCMP1545 (Table 1). Intriguingly, the draft genome of *Aureococcus anophagefferens*, despite its larger size (~56 Mb), also appears to lack homologs of key RNAi components (Table 1), although caution is required in this interpretation since ~10% of the genome is estimated to remain unsequenced (31). These observations are consistent with the proposal that the RNAi machinery appeared very early during eukaryotic evolution, but it seems to have been lost independently in several eukaryotic lineages (14, 63).

AGO-Piwi proteins are clearly identifiable in many algal species, including those, when genomic information is available, where RNAi-related phenomena have been experimentally demonstrated (Table 1). Green algae appear to have exclusively AGO proteins (11), as is the case for land plants (14, 37). However, the AGO-Piwi proteins from some chromalveolata algae are too divergent to be assigned with confidence to the AGO or Piwi clades (22). Dicer homologs are not as easily recognizable as AGO-Piwi proteins (Table 1), even in algal species with verified RNAi, such as the diatom *Phaeodactylum tricornutum* (22). As previously discussed (14), the multidomain structure of the Dicer enzymes of higher eukaryotes is not well conserved among protists (Fig. 1) and an RNase III domain may be all that is strictly required for function (1, 23, 71). Lastly, RDR-related proteins appear to have a more limited taxonomic distribution among the algae sampled (Table 1), consistent with an ancillary role in standard RNAi (14). Yet, their presence in certain species may allow transitive RNAi (i.e., the spreading of silencing to regions outside that initially targeted by a dsRNA trigger) (15, 89) and may increase the likelihood of undesirable off-target effects in reverse genetics studies (33). Additional components of the RNAi machinery in algae, to our knowledge, have been characterized in only one species, the unicellular photoautotroph *Chlamydomonas reinhardtii* (11, 40, 41, 46, 98).

The occurrence of sRNAs, potentially generated by Dicer-mediated processing, has been examined in very few algae (Table 1). *C. reinhardtii* has been shown to contain a complex set of endogenous sRNAs, including miRNAs, phased siRNAs, and siRNAs originating from transposons and repeated DNA regions (41, 61, 102). The production of siRNAs from IR transgenic transcripts and of artificial miRNAs (amiRNAs) from transgenes mimicking the structure of miRNA precursors has also been demonstrated in *Chlamydomonas* (40, 62, 76, 103). Likewise, endogenous noncoding sRNAs have been identified, by sRNA library sequencing, in the red alga *Porphyra yezoensis* (52), the diatom *P. tricornutum* (22), and the brown alga *Ectocarpus siliculosus* (17). Based on the ability of genomic sRNA loci (or of the corresponding transcripts) to fold back into hairpin secondary structures resembling miRNA precursors, miRNA candidates have been predicted in these three algal species (17, 52, 55). Interestingly, with the possible exception of candidate miRNAs identified in *P. yezoensis* (52), there appears to be little conservation of particular miRNA genes among algae or with those encoded in the genomes of land plants or animals (17, 18, 61, 102). This suggests, as already proposed for metazoans and higher plants (5, 14, 18),

that (many) miRNA genes and their potential regulatory interaction(s) with target mRNAs may have evolved independently in specific eukaryotic lineages.

dsRNA-induced gene silencing, suggestive of a functional RNAi machinery, has also been demonstrated in a limited number of algal species (Table 1), including the green algae *C. reinhardtii*, *Dunaliella salina*, and *Volvox carteri*; the diatom *P. tricornutum*; the yellow-green alga *Vaucheria frigida*; and the unicellular flagellate *Euglena gracilis* (16, 22, 24, 42, 43, 44, 76, 77, 79, 87). In most cases, reduction of the steady-state levels of targeted mRNAs was observed, implying RNAi-mediated transcriptional repression and/or RNA degradation (16, 22, 42, 43, 44, 76, 77, 79, 87). However, in *P. tricornutum*, introduction of an IR transgene, producing dsRNA homologous to a phytochrome gene, did not alter target transcript levels but significantly reduced cognate protein abundance, as detected by immunoblotting assays (22). Thus, as reported in animals and land plants (25, 39, 92, 97), RNA-mediated silencing may also operate by translation repression in the diatom *P. tricornutum*. We have made similar observations in the green alga *C. reinhardtii*, where RNAi triggered by IR transgenes may result in gene silencing by target transcript degradation and/or by translation repression (unpublished data).

The presence of key components of the RNAi machinery and of endogenous miRNAs/siRNAs, as well as the observation of experimentally triggered RNAi in algal species from three eukaryotic supergroups, suggests that RNA-mediated silencing may play regulatory roles and may be used as a reverse genetics tool in a wide spectrum of algae. However, it is also becoming apparent that in several species, particularly those with small nuclear genomes, the RNAi machinery seems to have been entirely lost or extensively modified. As Dicer-like proteins can be quite divergent and RDRs are not essential for RNA-mediated silencing (14, 83), the presence of a conserved AGO-Piwi protein may be diagnostic of the existence of the RNAi pathway in newly sequenced genomes. Yet, caution is also required in judging the significance of a putative RNAi machinery for reverse genetics studies, since the fungal pathogen *Candida albicans*, for instance, encodes an AGO homolog and a divergent Dicer protein (23) capable of processing long dsRNAs to siRNAs but fails to show RNAi against an endogenous gene when triggered by hairpin dsRNA produced by an IR transgene (83).

BIOLOGICAL ROLES OF RNA-MEDIATED SILENCING IN ALGAE

In eukaryotes, sRNA-mediated processes have been implicated in transposon silencing, resistance to viruses, regulation of endogenous gene expression, heterochromatin formation, DNA methylation, programmed DNA elimination, and maintenance of genome stability (10, 14, 15, 30, 36, 45, 49, 54, 64, 91, 92). However, not all of these phenomena occur in every single species. Ancestral functions of the RNAi machinery, such as defense responses against genomic parasites, may be shared by most eukaryotes, whereas other functions, such as programmed DNA elimination in ciliated protozoa, may have evolved more recently within specific lineages (14). Additionally, RNAi does not appear to be essential for cellular life since core components of the machinery have been lost in multiple taxonomically divergent eukaryotes (14, 23, 63). The possibility that an essential RNAi role(s) was compensated by an alternative mechanism(s) in these species seems to be a less parsimonious proposition since, given the widespread phyletic distribution of organisms lacking RNAi (14, 23, 63), such a mechanism(s) would have had to evolve independently multiple times.

In land plants, metazoans, and filamentous fungi, duplication and diversification of genes encoding proteins implicated in sRNA biogenesis and/or RNAi effector functions have given rise to distinct, partly overlapping pathways involved in the control of gene expression, suppression of genomic parasites, or modifications of DNA/chromatin (10, 14, 15, 30, 45, 51, 64, 92). This functional diversification is just beginning to be elucidated, but the presence of duplicated RNAi components appears to allow the evolution of novel gene control mechanisms that use sRNAs as sequence-specific determinants and more effective strategies to counteract the action of invading viruses and transposable elements (10, 14, 15, 30, 45, 51, 64, 92). In contrast to this wealth of information, relatively little is known about the biological role(s) and possible pathway(s) of RNA-mediated silencing in algae.

RNAi has been implicated in the repression of transposable elements in many eukaryotes (14, 15, 51, 54, 64), and it likely performs a similar function in algal species. The green alga *Chlamydomonas reinhardtii* has undergone duplication of key RNAi components (11) and contains three Dicer and three Argonaute proteins (Table 1). One of the Dicer proteins, DCL1, is uniquely required for the posttranscriptional silencing of retrotransposons, a function that is not compensated by the other two Dicer homologs encoded in the genome (11). Evidence suggests that DCL1 (and presumably one of the Argonaute proteins, AGO1) may be part of a siRNA pathway that is specialized for the control of transposable elements. However, *C. reinhardtii* also relies on a DCL1-independent transcriptional silencing mechanism(s) for the maintenance of transposon repression (11, 12, 80, 90). Interestingly, this chromatin-mediated silencing is sensitive to temperature, being much more effective at 17°C than at 25°C (13). Conversely, posttranscriptional gene repression by RNAi, in both invertebrates and land plants, appears to be more efficient at 25 to 29°C than at lower temperatures (28, 86). Thus, in *C. reinhardtii*, multiple silencing mechanisms, some RNAi dependent and some RNAi independent, may allow more effective control of transposon mobilization over a wide range of environmental conditions.

Consistent with a role for RNAi in transposon repression in algae, sRNAs mapping to transposable elements or low-complexity or simple repeat regions have been identified by high-throughput sequencing in *C. reinhardtii* and *E. siliculosus* (17, 41, 61, 102). Repeated sequences, including DNA transposons, retrotransposons, and helitrons, make up 22.7% of the *Ectocarpus* genome (17), and as in *Chlamydomonas* (11), RNAi may contribute to the silencing of these elements. The majority of the sequenced sRNAs in the red alga *P. yezoensis* remain unannotated because of the lack of genomic information (52). However, it is tempting to speculate that a fraction of these sRNAs may also correspond to siRNAs involved in transposon repression (72, 101).

In the diatom *P. tricornutum*, RNA-mediated silencing of a

β-glucuronidase (GUS) transgene induced DNA methylation of the target gene, which spreads, as observed in land plants (19, 89), to transcribed sequences beyond the region of complementarity to the trigger dsRNA (22). Interestingly, cytosine DNA methylation has also been detected in association with *P. tricornutum* transposable elements (58). Some of these retrotransposons are transcriptionally activated in response to environmental stress such as nitrogen starvation, correlating with changes in DNA methylation, and may promote genome rearrangements contributing to diatom diversification (58). Conceivably, RNAi mechanisms of transcriptional gene silencing (in particular, RNA-directed DNA methylation) and/or posttranscriptional gene silencing may prevent or modulate the transposon mobilization occurring in diatoms. The observations in *P. tricornutum* also underline mechanistic differences in RNAi pathways among widely divergent algal species. In *C. reinhardtii*, silencing of an *aadA* transgene with a dsRNA-producing IR construct does not result in DNA methylation of the target gene (100), suggesting that, unlike the case in diatoms, sRNAs may not direct methylation of *trans* complementary cDNA sequences in this green alga.

The limited information discussed above does suggest that RNAi plays a role in defense responses to transposable elements in algae, whereas a possible function in antiviral immunity remains unexplored. Another major role of RNAi in higher eukaryotes is the regulation of endogenous gene expression by miRNAs and other sRNAs (5, 10, 18, 30, 36, 45, 49, 92). As already mentioned, candidate miRNAs have been identified or predicted in four algal species, *C. reinhardtii* (41, 61, 102), *P. yezoensis* (52), *P. tricornutum* (22, 55), and *E. siliculosus* (17). To address the biological role(s) of these miRNAs, computational approaches have been used to predict their putative targets in three algae (17, 52, 61, 102). However, since the genomes of these species are not fully annotated or are not available (candidate miRNAs were matched only to expressed sequence tags for *P. yezoensis*) and/or many genes have no known function, it is difficult to draw a conclusion as to whether predicted miRNA targets have any functional bias. An additional constraint is that target gene identification is a challenging problem (88) and the false-positive prediction rate for miRNA targets in algae is not known. With the caveats posed by these uncertainties, predicted miRNA targets in both *C. reinhardtii* and *P. yezoensis* appear to correspond predominantly to genes involved in cellular metabolism and physiological processes, whereas transcription factors seem to be underrepresented (52, 61, 102). Interestingly, in *Chlamydomonas*, the steady-state levels of certain miRNAs change substantially under nitrogen starvation (61, 102) and they may play a role in (directly or indirectly) modulating lipid metabolism under these conditions, since mutants defective in miRNA biogenesis show reduced accumulation of triacylglycerols (unpublished data).

In land plants and metazoans, miRNAs are involved in the regulation of developmental programs and may have played an important role in the evolution of complex multicellularity (5, 18, 36, 45, 73, 92). Intriguingly, in the filamentous brown alga *Ectocarpus siliculosus*, which is closely related to the kelps, the majority of the predicted miRNA targets encode proteins with leucine-rich repeat (LRR) domains (17). These LRR-encoding genes include many members of the ROCO (Roc GTPase plus COR domain) family, envisaged to have evolved after the evolutionary split from the diatoms (17). Thus, a major proportion of the candidate miRNAs identified in *E. siliculosus* may regulate recently evolved processes potentially involved in the transition of brown algae to multicellularity (17).

Most characterized land plant miRNAs appear to regulate transcripts with highly complementary binding sites and trigger Argonaute-mediated endonucleolytic cleavage of target mRNAs (5, 10, 15, 25, 39, 92). Once the initial cut is made, cellular exonucleases complete the degradation of the resulting mRNA fragments (66, 82). In contrast, imperfect miRNA-mRNA hybrids, with central bulges or mismatches typical of metazoans, generally result in translational inhibition and/or accelerated exonucleolytic ("slicer"-independent) mRNA decay (5, 10, 25, 39). However, recent evidence indicates that sRNAs perfectly complementary to a target transcript can also cause translational repression without, or with only minimal, mRNA destabilization (92, 97). In the alga *C. reinhardtii*, expected mRNA cleavage products for a few predicted miRNA targets have been detected by 5′ rapid amplification of cDNA ends (61, 102), indicating that miRNAs can trigger endonucleolytic cleavage, as observed in land plants. However, for many other predicted miRNA targets, RNA products with cleavage sites located within the miRNA pairing region have not been observed (61, 102), suggesting regulation by alternative means such as RNAi-mediated translation repression (which does occur in *Chlamydomonas*), differences in the turnover of cleavage products in comparison with land plants, and/or false-positive prediction of miRNA targets. In other algal species, the mode of action of miRNAs has not been examined, to our knowledge.

As emphasized by this brief discussion, the natural roles of RNAi in algae are poorly understood. Limited evidence suggests that a siRNA pathway operates as a defense mechanism against transposon mobilization in several algal species. A miRNA pathway, when present, may contribute to regulatory responses to nutrient stress in unicellular algae, whereas it may also play a potential role in developmental programs associated with multicellular species. Other possible functions of RNAi, such as resistance to viruses, heterochromatin formation, or maintenance of genome stability, remain to be explored in these organisms. Given the significance of RNA-mediated silencing as a gene regulatory mechanism in higher eukaryotes, it will be of great interest to examine whether sRNAs may play key roles in sexual and/or asexual reproduction, responses to a variety of environmental stresses and/or the control of metabolic pathways in a wide range of phylogenetically diverse algae.

RNAi AS A GENETIC TOOL IN ALGAL BIOLOGY

Complete or near-complete genome sequences have been reported for several algae (3, 6, 7, 17, 21, 31, 57, 59, 68, 70, 74, 95, 96). Yet, lack of knowledge regarding gene function and regulatory processes is a serious impediment to our understanding of algal biology. Many predicted protein-encoding genes (~30% or a larger percentage) have no known function or are only poorly characterized (96), and noncoding RNAs remain virtually unexplored in most algae. To address this challenging problem, efforts have been made in recent years to

develop techniques for genetic transformation and functional genomic analyses in algal species (29, 32, 34, 70, 75, 96). Reverse genetics strategies, such as insertional mutagenesis and targeted gene disruption, have been used to analyze gene function in a few algae (20, 29, 60, 104, 105). However, many of these approaches are laborious, requiring extensive screening to identify specific gene mutations, and/or limited by biological constraints. For instance, the direct manipulation of target genes by homologous recombination has proven difficult even in the very few algae where the approach has met with some success (60, 65, 104, 105) and the generation of loss-of-function mutants by insertional or chemical mutagenesis may be challenging in diploid organisms with unknown sexual cycles such as some diatoms (70). As an alternative, RNA-mediated silencing is becoming a promising tool for targeted gene knockdown in algae having a functional RNAi machinery.

RNAi against specific genes can be induced by the introduction of exogenously synthesized dsRNAs or siRNAs into cells or whole organisms (33, 35, 42, 43, 87). Nonintegrative dsRNA/siRNA-producing viral vectors or plasmids can also be used to trigger temporary RNAi (33, 35, 85, 93). These approaches generally result in transient, not heritable gene repression effects. Within algal species, *in vitro*-synthesized long dsRNAs have been electroporated into *Euglena gracilis* cells and shown to silence successfully, with demonstrable reduction of mRNA levels, two endogenous genes homologous to the introduced dsRNAs (42, 43). The xanthophycean algae in the genus *Vaucheria* consist of sparsely branched, multinucleate tube cells without septal walls (87). Exogenously synthesized dsRNA has been microinjected into the giant multinucleate cells of *V. frigida* to suppress the expression of a blue light photoreceptor (87). Interestingly, the elicited RNAi effect appeared to last longer than 6 months based on the persistence of induced abnormal phenotypes such as retarded tip growth and altered tube morphology (87). In the green alga *Dunaliella salina*, plasmid DNA containing an IR transgene, which produced hairpin dsRNA homologous to the phytoene desaturase gene, was introduced into cells by electroporation and shown to reduce target mRNA levels maximally at 7 days after electroporation (85). These techniques of transient suppression of gene expression with *in vitro*-synthesized dsRNAs or transgenic DNA constructs may be broadly applicable to algae, even if a stable transformation protocol and selectable markers are not available. All that is needed is a method to deliver nucleic acids (RNA and/or DNA) efficiently into cells and/or a way (usually expression of a cotransfected reporter gene) to differentiate the cells that have taken up the foreign macromolecules for phenotypic evaluation.

Stable and heritable RNAi has also been developed in several algae with available genome transformation methodologies (22, 44, 46, 47, 62, 76, 77, 78, 79, 103). Common approaches rely on the production of antisense RNA or hairpin dsRNA by transcription from genome-integrated transgenes (16, 22, 44, 46, 47, 76, 77, 79). In these strategies, long dsRNAs, generated by the annealing of transgenic antisense and endogenous sense RNAs or by hairpin formation by complementary IRs in transgenic transcripts, are likely processed by Dicer and subsequently enter the siRNA pathway. In *Volvox carteri* and *C. reinhardtii*, antisense or IR transgenic approaches have been proven successful at downregulating the expression of numerous genes (16, 24, 46. 47, 76, 79), although IR transgenes seem to be more effective at achieving higher degrees of suppression (79). An IR construct stably introduced into the nuclear genome by electroporation has also been used to silence the expression of the glyceraldehyde-3-phosphate dehydrogenase gene in *D. salina* (44). Gene silencing was also triggered in the diatom *P. tricornutum* using transgenes stably integrated into the genome and containing either antisense or IR sequences complementary to a GUS reporter or the endogenous phytochrome, cryptochrome, or UMP synthase genes (22, 77). Antisense and IR constructs proved to be equally effective at suppressing GUS expression (22), although this diatom contains an RDR (Table 1) that may facilitate the conversion of antisense RNA to dsRNA.

One problem with these approaches is that the level of silencing triggered by genome integrated transgenes is often quite variable, depending on the type of construct, transgene copy number, site of integration, and target gene (33, 46, 76, 79, 93). Consequently, several (many) individual lines may need to be molecularly characterized for the suppression of a certain gene before potential phenotypic defects can be evaluated. In *Chlamydomonas reinhardtii*, IR transgenes producing long dsRNAs have the disadvantage that they may be prone to self-silencing at the transcriptional level (76, 100), although this may be partly avoided by expression from an inducible promoter (47). An additional issue with all RNAi approaches is target specificity, since transcripts partly complementary to the trigger dsRNAs or siRNAs may also be silenced unintentionally, a phenomenon termed the "off-target" effect (33, 48, 81, 99).

Two strategies have been developed in *C. reinhardtii* to overcome some of these problems. Transgenes mimicking the structure of miRNA precursors have been exploited to produce amiRNAs targeting genes of interest (62, 78, 103). The expression of these amiRNAs appears to be more stable than that of long dsRNAs produced from IR transgenes, and since each amiRNA precursor gives rise to a single sRNA species, they can be optimized to avoid off-target effects (62, 103). Several genes have been successfully downregulated with this approach, including *COX90* (encoding a cytochrome *c* oxidase subunit), the gene for phytoene synthase (involved in carotenoid biosynthesis), *DCL1*, *MAA7* (encoding the tryptophan synthase β subunit), and *RBCS1/2* (a small multigene family encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) (62, 103). The production of amiRNAs driven by an inducible promoter has also been achieved (78). However, there are also disadvantages of the amiRNA strategy since many aspects of sRNA processing, regulation, and miRNA action in *Chlamydomonas*, as well as in most other algae, remain unknown. Thus, designed amiRNAs may not be processed as intended or the unique site where an amiRNA would associate on a target transcript may not be accessible, for instance, due to being occluded by protein binding and/or by the formation of secondary RNA structures (33, 62). This may result in the accumulation of ineffective amiRNAs with certain transgenes and may necessitate the design of several amiRNA constructs for a given target gene (62).

An alternative approach has been the development of a tandem IR RNAi system that allows for the direct selection of effective transgenic RNAi lines and the simultaneous

silencing of two unrelated genes (46, 76). In *Chlamydomonas*, the *MAA7*-encoded tryptophan synthase β subunit converts the indole analog 5-fluoroindole (5-FI) into the toxic tryptophan analog 5-fluorotryptophan. RNAi-mediated suppression of *MAA7* leads to strains resistant to 5-FI (46, 76), although selection conditions need to be carefully established due to the inactivation of 5-FI by light exposure. Direct selection for this RNAi-induced phenotype permits the identification of transgenic lines showing effective interference of any (nonessential) gene cotargeted with *MAA7*, as part of the same dsRNA trigger synthesized from a tandem IR (46, 76). Several genes have been shown to be cosuppressed with *MAA7*, including *TRXH1* (encoding cytosolic thioredoxin 1), *RBP1* (encoding RNA binding protein 1), *KU80* (involved in the repair of DNA double-strand breaks), and a gene coding for a predicted exoribonuclease with weak similarity to 3′hExo/ERI-1 (46, 76). Transcriptional self-silencing and other problems associated with genomic transgene integration are avoided by this selection strategy (76). Additionally, the design of tandem IR transgenes is simplified by the production of long dsRNAs that can be processed into a complex and heterogeneous population of siRNAs. This increases the probability, relative to a single amiRNA, that at least some of the siRNAs generated will be assembled into functional effector complexes and will be able to access the target transcript. The greater risk of off-target effects when using long dsRNAs can be circumvented by silencing each gene of interest with at least two separate IR transgenes, homologous to distinct and nonoverlapping sequences of the target gene (46). If similar phenotypes were found in several independent RNAi strains generated with different transgenes, it would increase confidence that the observed phenotypic abnormalities are the consequence of downregulation of the intended gene (46). In addition, correlation between target transcript and/or protein depletion and phenotypic severity should also be established.

Another strategy to verify the specificity of RNAi-induced phenotypes was employed in the diatom *P. tricornutum*. The endogenous UMP synthase gene was initially silenced by nuclear integration of an IR transgene targeting the 5′ region of the mRNA, which resulted in cells requiring uracil and resistant to 5-fluoroorotic acid (5-FOA) (77). RNAi strains were then transformed with the coding sequence of the human UMP synthase gene, codon optimized, and driven by *P. tricornutum* regulatory regions. The transformants showed reversion of the RNAi-induced phenotypes becoming autotrophs for uracil and sensitive to 5-FOA (77). This confirmed that the observed RNAi-induced phenotypes were due to downregulation of the expression of the diatom UMP synthase gene. However, this "phenotype reversion" strategy is dependent on finding a heterologous gene (or alternatively generating a synthetic one) with enough nucleotide sequence divergence so that it is not targeted by RNAi triggered against an endogenous algal homolog while retaining enough conservation at the amino acid level for functional complementation. Inhibition of RNAi by virus-encoded RNA-silencing suppressors can also be exploited to evaluate whether an induced phenotype is dependent on sRNA silencing pathways (2). Despite substantial recent progress in the use of RNA-mediated silencing approaches in algae, developments are still needed in the design of high-throughput methods for the analysis of gene function, such as RNAi screens (33, 48, 81), as well as the design of improved RNAi vectors, for instance, involving strategies akin to virus-induced gene silencing in higher plants (89, 93).

PERSPECTIVE

Algae encompass eukaryotic organisms from very diverse phylogenetic lineages (4, 9, 69, 70, 96) with distinctive physiologies and ecologies (70, 96), emphasizing the impossibility of defining a single algal species as a model for the whole group. The era of algal genomics has just begun, but the few available genomes are already providing significant insights into the complexity of these organisms (34, 70, 96). Genomic information is also illuminating our lack of knowledge of the biological functions of a significant fraction of the predicted protein-encoding genes (59, 96) and of the gene regulatory mechanisms operating in most algae.

RNA-mediated silencing processes have been implicated in transposon repression and endogenous gene regulation in a few algal species. However, other possible functions of RNAi, such as resistance to viruses, heterochromatin formation, or maintenance of genome stability, remain to be examined. More broadly, the contributions of other epigenetic mechanisms to gene regulation are largely uncharacterized in algae, and their considerable evolutionary divergence from animal, plant, and fungal species makes it difficult to extrapolate the knowledge acquired from these well-characterized model systems. It is tempting to postulate that RNA-mediated silencing and other epigenetic processes may confer on algal species phenotypic plasticity in their adaptation to fluctuating environmental conditions. Examining these mechanisms in key lineages may contribute substantially to our understanding of the diversity of algal physiological responses and hint at the evolutionary basis for this divergence. Additionally, the genetic changes that brought about the evolution of multicellular life from unicellular progenitors remain obscure. It may be worth exploring whether distinctive miRNA-mediated gene regulation emerges as a basic feature in algal lineages that have evolved independently multicellular stages.

From a practical perspective, RNAi is becoming a customary method for directed gene silencing in algae. As the necessary molecular tools are developed, RNAi approaches are expected to contribute to the functional characterization of novel genes, as well as to the genetic engineering of algae. However, potential drawbacks to this promising technology are also apparent, such as a possible lack of specificity resulting in off-target effects and variations in the degree of induced silencing for particular genes. This emphasizes the need for careful design of RNAi experiments, including appropriate negative controls and the use of multiple RNAi reagents (dsRNAs, siRNAs, amiRNAs, or IR-produced hairpin dsRNAs) for each target gene. The verification of RNAi results and the validation of their biological relevance, whenever possible by using alternative methods, are also important steps in any experimental design. Ultimately, RNAi technology, in conjunction with system level "omics" approaches, may provide much-needed insights into gene function, metabolic pathways, and regulatory networks to allow us to comprehend the role of algal species in nature, as well as to engineer these organisms for the synthesis of valuable bioproducts.

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REFERENCES

1. **Abed, M., and S. Ankri.** 2005. Molecular characterization of *Entamoeba histolytica* RNaseIII and AGO2, two RNA interference hallmark proteins. Exp. Parasitol. **110:**265–269.
2. **Ahn, J. W., C. J. Yin, J. R. Liu, and W. J. Jeong.** 2010. Cucumber mosaic virus 2b protein inhibits RNA silencing pathways in green alga *Chlamydomonas reinhardtii*. Plant Cell Rep. **29:**967–975.
3. **Armbrust, E. V., et al.** 2004. The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. Science **306:**79–86.
4. **Baldauf, S. L.** 2008. An overview of the phylogeny and diversity of eukaryotes. J. Syst. Evol. **46:**263–273.
5. **Bartel, D. P.** 2009. MicroRNAs: target recognition and regulatory functions. Cell **136:**215–233.
6. **Blanc, G., et al.** 2010. The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex. Plant Cell **22:**2943–2955.
7. **Bowler, C., et al.** 2008. The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. Nature **456:**239–244.
8. **Boyce, D. G., M. R. Lewis, and B. Worm.** 2010. Global phytoplankton decline over the past century. Nature **466:**591–596.
9. **Burki, F., K. Shalchian-Tabrizi, and J. Pawlowski.** 2008. Phylogenomics reveals a new 'megagroup' including most photosynthetic eukaryotes. Biol. Lett. **4:**366–369.
10. **Carthew, R. W., and E. J. Sontheimer.** 2009. Silence from within: endogenous siRNAs and miRNAs. Cell **136:**642–655.
11. **Casas-Mollano, J. A., et al.** 2008. Diversification of the core RNA interference machinery in *Chlamydomonas reinhardtii* and the role of DCL1 in transposon silencing. Genetics **179:**69–81.
12. **Casas-Mollano, J. A., B. R. Jeong, J. Xu, H. Moriyama, and H. Cerutti.** 2008. The MUT9p kinase phosphorylates histone H3 threonine 3 and is necessary for heritable epigenetic silencing in *Chlamydomonas*. Proc. Natl. Acad. Sci. U. S. A. **105:**6486–6491.
13. **Cerutti, H., A. M. Johnson, N. W. Gillham, and J. E. Boynton.** 1997. Epigenetic silencing of a foreign gene in nuclear transformants of *Chlamydomonas*. Plant Cell **9:**925–945.
14. **Cerutti, H., and J. A. Casas-Mollano.** 2006. On the origin and functions of RNA-mediated silencing: from protists to man. Curr. Genet. **50:**81–99.
15. **Chapman, E. J., and J. C. Carrington.** 2007. Specialization and evolution of endogenous small RNA pathways. Nat. Rev. Genet. **8:**884–896.
16. **Cheng, Q., A. Hallmann, L. Edwards, and S. M. Miller.** 2006. Characterization of a heat-shock-inducible hsp70 gene of the green alga *Volvox carteri*. Gene **371:**112–120.
17. **Cock, J. M., et al.** 2010. The *Ectocarpus* genome and the independent evolution of multicellularity in brown algae. Nature **465:**617–621.
18. **Cuperus, J. T., N. Fahlgren, and J. C. Carrington.** 2011. Evolution and functional diversification of miRNA genes. Plant Cell **23:**431–442.
19. **Daxinger, L., et al.** 2009. A stepwise pathway for biogenesis of 24-nt secondary siRNAs and spreading of DNA methylation. EMBO J. **28:**48–57.
20. **Dent, R. M., C. M. Haglund, B. L. Chin, M. C. Kobayashi, and K. K. Niyogi.** 2005. Functional genomics of eukaryotic photosynthesis using insertional mutagenesis of *Chlamydomonas reinhardtii*. Plant Physiol. **137:**545–556.
21. **Derelle, E., et al.** 2006. Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. Proc. Natl. Acad. Sci. U. S. A. **103:**11647–11652.
22. **De Riso, V., et al.** 2009. Gene silencing in the marine diatom *Phaeodactylum tricornutum*. Nucleic Acids Res. **37:**e96.
23. **Drinnenberg, I. A., et al.** 2009. RNAi in budding yeast. Science **326:**544–550.
24. **Ebnet, E., M. Fischer, W. Deininger, and P. Hegemann.** 1999. Volvoxrhodopsin, a light-regulated sensory photoreceptor of the spheroidal green alga *Volvox carteri*. Plant Cell **11:**1473–1484.
25. **Fabian, M. R., N. Sonenberg, and W. Filipowicz.** 2010. Regulation of mRNA translation and stability of microRNAs. Annu. Rev. Biochem. **79:**351–379.
26. **Field, C. B., M. J. Behrenfeld, J. T. Randerson, and P. Falkowski.** 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. Science **281:**237–240.
27. **Fire, A., et al.** 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature **391:**806–811.
28. **Fortier, E., and J. M. Belote.** 2000. Temperature-dependent gene silencing by an expressed inverted repeat in *Drosophila*. Genesis **26:**240–244.
29. **Galván, A., D. Gonzales-Ballester, and E. Fernandez.** 2007. Insertional mutagenesis as a tool to study genes/functions in *Chlamydomonas*. Adv. Exp. Med. Biol. **616:**77–89.
30. **Ghildiyal, M., and P. D. Zamore.** 2009. Small silencing RNAs: an expanding universe. Nat. Rev. Genet. **10:**94–108.
31. **Gobler, C. J., et al.** 2011. Niche of harmful alga *Aureococcus anophagefferens* revealed through ecogenomics. Proc. Natl. Acad. Sci. U. S. A. **108:**4352–4357.
32. **González-Ballester, D., et al.** 2010. RNA-Seq analysis of sulfur-deprived *Chlamydomonas* cells reveals aspects of acclimation critical for cell survival. Plant Cell **22:**2058–2084.
33. **Grimm, D.** 2009. Small silencing RNAs: state-of-the-art. Adv. Drug Deliv. Rev. **61:**672–703.
34. **Grossman, A. R., et al.** 2007. Novel metabolism in *Chlamydomonas* through the lens of genomics. Curr. Opin. Plant Biol. **10:**190–198.
35. **Hebert, C. G., J. J. Valdes, and W. F. Bentley.** 2008. Beyond silencing—engineering applications of RNA interference and antisense technology for altering cellular phenotype. Curr. Opin. Biotechnol. **19:**500–505.
36. **Herranz, H., and S. M. Cohen.** 2010. MicroRNAs and gene regulatory networks: managing the impact of noise in biological systems. Genes Dev. **24:**1339–1344.
37. **Höck, J., and G. Meister.** 2008. The Argonaute protein family. Genome Biol. **9:**210.
38. **Hu, Q., et al.** 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. **54:**621–639.
39. **Huntzinger, E., and E. Izaurralde.** 2011. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat. Rev. Genet. **12:**99–110.
40. **Ibrahim, F., J. Rohr, W.-J. Jeong, J. Hesson, and H. Cerutti.** 2006. Untemplated oligoadenylation promotes degradation of RISC-cleaved transcripts. Science **314:**1893.
41. **Ibrahim, F., et al.** 2010. Uridylation of mature miRNAs and siRNAs by the MUT68 nucleotidyltransferase promotes their degradation in *Chlamydomonas*. Proc. Natl. Acad. Sci. U. S. A. **107:**3906–3911.
42. **Iseki, M., et al.** 2002. A blue-light-activated adenylyl cyclase mediates photoavoidance in *Euglena gracilis*. Nature **415:**1047–1051.
43. **Ishikawa, T., et al.** 2008. The pathway via D-galacturonate/L-galactonate is significant for ascorbate biosynthesis in *Euglena gracilis*. J. Biol. Chem. **283:**31133–31141.
44. **Jia, Y., L. Xue, H. Liu, and J. Li.** 2009. Characterization of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene from the halotolerant alga *Dunaliella salina* and inhibition of its expression by RNAi. Curr. Microbiol. **58:**426–431.
45. **Ketting, R. F.** 2011. The many faces of RNAi. Dev. Cell **20:**148–161.
46. **Kim, E.-J., and H. Cerutti.** 2009. Targeted gene silencing by RNA interference in *Chlamydomonas*. Methods Cell Biol. **93:**99–110.
47. **Koblenz, B., and K. F. Lechtreck.** 2005. The NIT1 promoter allows inducible and reversible silencing of centrin in *Chlamydomonas reinhardtii*. Eukaryot. Cell **4:**1959–1962.
48. **Kulkarni, M. M., et al.** 2006. Evidence of off-target effects associated with long dsRNAs in *Drosophila melanogaster* cell-based assays. Nat. Methods **3:**833–838.
49. **Leung, A. K., and P. A. Sharp.** 2010. MicroRNA functions in stress responses. Mol. Cell **40:**205–215.
50. **Li, L., et al.** 2006. Small dsRNAs induce transcriptional activation in human cells. Proc. Natl. Acad. Sci. U. S. A. **103:**17337–17342.
51. **Li, L., S. S. Chang, and Y. Liu.** 2010. RNA interference pathways in filamentous fungi. Cell. Mol. Life Sci. **67:**3849–3863.
52. **Liang, C., et al.** 2010. Identification of miRNA from *Porphyra yezoensis* by high-throughput sequencing and bioinformatics analysis. PLoS One **5:**e10698.
53. **Liu, Q., and Z. Paroo.** 2010. Biochemical principles of small RNA pathways. Annu. Rev. Biochem. **79:**295–319.
54. **Malone, C. D., and G. J. Hannon.** 2009. Small RNAs as guardians of the genome. Cell **136:**656–668.
55. **Mathelier, A., and A. Carbone.** 2010. MIReNA: finding microRNAs with high accuracy and no learning at genome scale and from deep sequencing data. Bioinformatics **26:**2226–2234.
56. **Matsuyama-Serisawa, K., et al.** 2007. DNA content of the cell nucleus in the macroalga *Porphyra yezoensis* (Rhodophyta). Fish. Sci. **73:**738–740.
57. **Matsuzaki, M., et al.** 2004. Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D. Nature **428:**653–657.
58. **Maumus, F., et al.** 2009. Potential impact of stress activated retrotransposons on genome evolution in a marine diatom. BMC Genomics **10:**624.
59. **Merchant, S. S., et al.** 2007. The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science **318:**245–251.
60. **Minoda, A., R. Sakagami, F. Yagisawa, T. Kuroiwa, and K. Tanaka.** 2004. Improvement of culture conditions and evidence for nuclear transformation by homologous recombination in a red alga *Cyanidioschyzon merolae* 10D. Plant Cell Physiol. **45:**667–671.
61. **Molnár, A., F. Schwach, D. J. Studholme, E. C. Thuenemann, and D. C. Baulcombe.** 2007. miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii*. Nature **447:**1126–1129.
62. **Molnár, A., et al.** 2009. Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. Plant J. **58:**165–174.

63. **Nakayashiki, H., N. Kadotani, and S. Mayama.** 2006. Evolution and diversification of RNA silencing proteins in fungi. J. Mol. Evol. **63:**127–135.
64. **Obbard, D. J., K. H. Gordon, A. H. Buck, and F. M. Jiggins.** 2009. The evolution of RNAi as a defence against viruses and transposable elements. Philos. Trans. R. Soc. Lond. B Biol. Sci. **364:**99–115.
65. **Ohnuma, M., et al.** 2009. Transient gene suppression in a red alga, Cyanidioschyzon merolae 10D. Protoplasma **236:**107–112.
66. **Orban, T. I., and E. Izaurralde.** 2005. Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. RNA **11:**459–469.
67. **Pak, J., and A. Fire.** 2007. Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. Science **315:**241–244.
68. **Palenik, B., et al.** 2007. The tiny eukaryote *Ostreococcus* provides genomic insights into the paradox of plankton speciation. Proc. Natl. Acad. Sci. U. S. A. **104:**7705–7710.
69. **Parfrey, L. W., et al.** 2006. Evaluating support for the current classification of eukaryotic diversity. PLoS Genet. **2:**e220.
70. **Parker, M. S., T. Mock, and E. V. Armbrust.** 2008. Genomic insights into marine microalgae. Annu. Rev. Genet. **42:**619–645.
71. **Patrick, K. L., et al.** 2009. Distinct and overlapping roles of two Dicer-like proteins in the RNA interference pathways of the ancient eukaryote *Trypanosoma brucei*. Proc. Natl. Acad. Sci. U. S. A. **106:**17933–17938.
72. **Peddigari, S., W. Zhang, K. Takechi, H. Takano, and S. Takio.** 2008. Two different clades of copia-like retrotransposons in the red alga, *Porphyra yezoensis*. Gene **424:**153–158.
73. **Peterson, K. J., M. R. Dietrich, and M. A. McPeek.** 2009. MicroRNAs and metazoan macroevolution: insights into canalization, complexity, and the Cambrian explosion. Bioessays **31:**736–747.
74. **Prochnik, S. E., et al.** 2010. Genomic analysis of organismal complexity in the multicellular green alga *Volvox carteri*. Science **329:**223–226.
75. **Radakovits, R., R. E. Jinkerson, A. Darzins, and M. C. Posewitz.** 2010. Genetic engineering of algae for biofuel production. Eukaryot. Cell **9:**486–501.
76. **Rohr, J., N. Sarkar, S. Balenger, B. R. Jeong, and H. Cerutti.** 2004. Tandem inverted repeat system for selection of effective transgenic RNAi strains in *Chlamydomonas*. Plant J. **40:**611–621.
77. **Sakaguchi, T., K. Nakajima, and Y. Matsuda.** 2011. Identification of the UMP synthase gene by establishment of uracil auxotrophic mutants and the phenotypic complementation system in the marine diatom *Phaeodactylum tricornutum*. Plant Physiol. **156:**78–89.
78. **Schmollinger, S., D. Strenkert, and M. Schroda.** 2010. An inducible artificial microRNA system for *Chlamydomonas reinhardtii* confirms a key role for heat shock factor 1 in regulating thermotolerance. Curr. Genet. **56:**383–389.
79. **Schroda, M.** 2006. RNA silencing in *Chlamydomonas*: mechanisms and tools. Curr. Genet. **49:**69–84.
80. **Shaver, S. S., J. A. Casas-Mollano, R. L. Cerny, and H. Cerutti.** 2010. Origin of the Polycomb Repressive Complex 2 and gene silencing by an E(z) homolog in the unicellular alga *Chlamydomonas*. Epigenetics **5:**301–312.
81. **Sigoillot, F. D., and R. W. King.** 2011. Vigilance and validation: keys to success in RNAi screening. ACS Chem. Biol. **6:**47–60.
82. **Souret, F. F., J. P. Kastenmayer, and P. J. Green.** 2004. AtXRN4 degrades mRNA in *Arabidopsis* and its substrates include selected miRNA targets. Mol. Cell **15:**173–183.
83. **Staab, J. F., T. C. White, and K. A. Marr.** 2011. Hairpin dsRNA does not trigger RNA interference in *Candida albicans* cells. Yeast **28:**1–8.
84. **Steitz, J. A., and S. Vasudevan.** 2009. miRNPs: versatile regulators of gene expression in vertebrate cells. Biochem. Soc. Trans. **37:**931–935.
85. **Sun, G., X. Zhang, Z. Sui, and Y. Mao.** 2008. Inhibition of pds gene expression via the RNA interference approach in *Dunaliella salina* (Chlorophyta). Mar. Biotechnol. **10:**219–226.
86. **Szittya, G., et al.** 2003. Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. EMBO J. **22:**633–640.
87. **Takahashi, F., et al.** 2007. AUREOCHROME, a photoreceptor required for photomorphogenesis in stramenopiles. Proc. Natl. Acad. Sci. U. S. A. **104:**19625–19630.
88. **Thomas, M., J. Lieberman, and A. Lai.** 2010. Desperately seeking microRNA targets. Nat. Struct. Mol. Biol. **17:**1169–1174.
89. **Vaistij, F. F., L. Jones, and D. C. Baulcombe.** 2002. Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell **14:**857–867.
90. **van Dijk, K. V., et al.** 2005. Monomethyl histone H3 lysine 4 as an epigenetic mark for silenced euchromatin in *Chlamydomonas*. Plant Cell **17:**2439–2453.
91. **van Wolfswinkel, J. C., and R. F. Ketting.** 2010. The role of small noncoding RNAs in genome stability and chromatin organization. J. Cell Sci. **123:**1825–1839.
92. **Voinnet, O.** 2009. Origin, biogenesis, and activity of plant microRNAs. Cell **136:**669–687.
93. **Waterhouse, P. M., and C. A. Helliwell.** 2003. Exploring plant genomes by RNA-induced gene silencing. Nat. Rev. Genet. **4:**29–38.
94. **Wijffels, R. H., and M. J. Barbosa.** 2010. An outlook on microalgal biofuels. Science **329:**796–799.
95. **Worden, A. Z., et al.** 2009. Green evolution and dynamic adaptations revealed by genomes of the marine picoeukaryotes *Micromonas*. Science **324:**268–272.
96. **Worden, A. Z., and A. E. Allen.** 2010. The voyage of the microbial eukaryote. Curr. Opin. Microbiol. **13:**652–660.
97. **Wu, L., J. Fan, and J. G. Belasco.** 2008. Importance of translation and nonnucleolytic Ago proteins for on-target RNA interference. Curr. Biol. **18:**1327–1332.
98. **Wu-Scharf, D., B. R. Jeong, C. Zhang, and H. Cerutti.** 2000. Transgene and transposon silencing in *Chlamydomonas reinhardtii* by a DEAH-box RNA helicase. Science **290:**1159–1162.
99. **Xu, P., Y. Zhang, L. Kang, M. J. Roossinck, and K. S. Mysore.** 2006. Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. Plant Physiol. **142:**429–440.
100. **Yamasaki, T., H. Miyasaka, and T. Ohama.** 2008. Unstable RNAi effects through epigenetic silencing of an inverted repeat transgene in *Chlamydomonas reinhardtii*. Genetics **180:**1927–1944.
101. **Zhang, W., et al.** 2007. Characterization of short interspersed elements (SINEs) in a red alga, *Porphyra yezoensis*. Biosci. Biotechnol. Biochem. **71:**618–622.
102. **Zhao, T., et al.** 2007. A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*. Genes Dev. **21:**1190–1203.
103. **Zhao, T., W. Wang, X. Bai, and Y. Qi.** 2009. Gene silencing by artificial microRNAs in *Chlamydomonas*. Plant J. **58:**157–164.
104. **Zorin, B., P. Hegemann, and I. Sizova.** 2005. Nuclear-gene targeting by using single-stranded DNA avoids illegitimate DNA integration in *Chlamydomonas reinhardtii*. Eukaryot. Cell **4:**1264–1272.
105. **Zorin, B., Y. Lu, I. Sizova, and P. Hegemann.** 2009. Nuclear gene targeting in *Chlamydomonas* as exemplified by disruption of the PHOT gene. Gene **432:**91–96.